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Sequence Variation of Hepatitis B Virus Precore-Core Open Reading Frame Isolated From Serum and Liver of Children With Chronic Hepatitis B Before and After Interferon Treatment

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DNA and amino acid sequences of the hepatitis B virus (HBV) genome were studied in serum and liver samples taken from 12 children with chronic hepatitis B before and after interferon (IFN) therapy. The purpose was to discover whether the persistence of low levels of viral replication with normal alanine aminotransferases after the response to IFN treatment is due to the appearance of mutations in the sequence of HB core antigen T and B cell epitopes. The existence of mutants was studied by amplification of precore-core region of the HBV genome by polymerase chain reaction (PCR) and direct sequencing of the PCR products. In addition to the wild type sequence, mutation 1896 in the precore region was detected in the baseline serum and liver samples of five children. No changes in the distribution were found in the final samples, except one case. In the core region, both the wild type sequence and amino acid substitutions were observed in the basal serum and/or liver samples of six patients and most of these remained detectable in the samples after treatment. Sixteen (67%) of 24 changes in the core amino acid sequences were found in the T- or B-cell epitopes. The results suggest that viral persistence after response to IFN therapy in children is not due to the appearance of mutants in the HBV core T- and B-cell epitopes and that the host immune response can control the viral replication. ***J. Med. Virol. 58: 208–214, 1999.***

KEY WORDS: IFN therapy; PCR; mutation; HBV-DNA; amino acid

INTRODUCTION

The hepatitis B virus (HBV) is a DNA virus that causes acute and chronic liver disease and its persistence is related to the development of hepatocellular carcinoma [Tiollais et al., 1985; Feitelson, 1992]. A vigorous, polyclonal and specific T-cell response against multiple HBV epitopes in the viral nucleocapsid, envelope, and polymerase proteins can be detected in the peripheral blood cells during acute HBV infection and may be responsible for viral clearance in patients with self-limited acute hepatitis [Ferrari et al., 1991; Bertoletti et al., 1993; Missale et al., 1993; Nayersina et al., 1993; Chisari and Ferrari, 1995; Rehermann et al., 1995].

Immune selection pressure exerted by the cytotoxic T lymphocytes (CTL) and T helper cell response could be responsible for viral persistence in patients with chronic infection. HBV sequence heterogeneity of hepatitis B core antigen (HBcAg) has been reported in chronically infected patients [Brunetto et al., 1990; Ehata et al., 1993; Carman et al., 1995]. Thus, it has been suggested that mutations in known HBcAg B and Human Leucocyte Antigen (HLA) class I- and class II-restricted T-cell epitopes [Salfeld et al., 1989; Ferrari et al., 1990, 1991; Bertoletti et al., 1991, 1993; Penna et al., 1991; Sällberg et al., 1991a, 1991b; Shödel et al., 1992; Missale et al., 1993; Seifer and Standring, 1993; Jung et al., 1994; Chisari and Ferrari, 1995] could be related to the viral persistence in chronic HBV carriers [Bertoletti et al., 1994; Carman et al., 1997; Torre and Naoumov, 1998].

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On the other hand, interferon (IFN) treatment is the most effective therapy for chronic hepatitis B virus infection. However, although the alanine aminotransferases (ALT) become normal in approximately 50% of the patients with therapy, most of them continue to have polymerase chain reaction (PCR)-detectable viral DNA in serum long after response to treatment [Di Bisceglie, 1995; López-Alcorocho et al., 1997].

In the current study, the complete DNA and amino acid sequences of the hepatitis B core antigen were compared in HBV-DNA isolated from serum and liver tissue samples taken from 12 children with chronic hepatitis B before and after IFN treatment to determine whether the persistence of low levels of viral replication in patients with normal ALT levels after IFN therapy can be related to the appearance of HBV mutants in the different T- and B-cell epitopes of the HBcAg.

MATERIALS AND METHODS

Patients

Serum and liver tissue samples taken from 12 children with chronic hepatitis B before and after IFN therapy were studied. The inclusion criterion was the availability of properly frozen paired serum and liver tissue samples. All patients had abnormal ALT levels (193 ± 159 IU/l; range: 50–660 IU/l) before IFN treatment. In the basal sample all patients had HBsAg and all but one had HBeAg (11/12, 92%). HBV-DNA was detected in serum by dot-blot hybridization in 11 of the patients (11/12,92%); the other patient was anti-HBe positive and HBV-DNA negative by dot-blot hybridization, but had HBV-DNA by PCR. On admission, liver histology showed minimal chronic hepatitis with mild fibrosis in three children, minimal chronic hepatitis without fibrosis in one, mild chronic hepatitis with mild fibrosis in four, moderate chronic hepatitis with mild fibrosis in two, mild chronic hepatitis with severe fibrosis in one, and moderate chronic hepatitis with cirrhosis in the remaining case. None of the children had antibodies against hepatitis C virus (anti-HCV) or human immunodeficiency virus (anti-HIV) but one (patient 3) had antibodies against hepatitis D virus (anti-HDV).

Regarding the source of HBV infection, perinatal transmission was well documented in five children. In five other children, household contact was the probable source of infection, as it was not the mother but other family members who were HBsAg-positive. In the remaining two children, the route of infection was unknown.

All the children were included in two different IFN-α trials; eight of them received doses of 10 MU/m² Body Surface (b.s.) of rIFN-α 2a (Roferon-A, Hoffmann-La Roche, Basel, Switzerland) for 6 or 9 months and the remaining four children were assigned to the control group of these trials. Response to treatment was defined as the loss of serum HBV-DNA as determined by dot-blot hybridization, ALT normalization and seroconversion to anti-HBe. Three of these eight children did not respond to the IFN-α therapy so they were included in a second treatment with IFN-β: 5 mU/m² b.s. of human derived fibroblast IFN-β (Serono Laboratories, Madrid, Spain) for 6 months; two of these three children responded to the IFN-β treatment and the remaining child was a nonresponder.

A second serum and liver tissue sample was taken from the 12 children during the follow-up of their respective treatments in a period ranging from 20 to 99 months (mean 61 ± 20 months) after the baseline samples had been taken. In the 3 children who were treated with IFN-α and then with IFN-β, the second sample was taken after the therapy with IFN-β. In the second sample, all but one child had normal ALT levels; 11/12 (92%) children had anti-HBe and 2 had lost the HBsAg and developed anti-HBs; one child continued to have HBeAg, was serum HBV-DNA positive by dot-blot hybridization, and had abnormal ALT levels. Histology of the second liver biopsy showed minimal chronic hepatitis with mild fibrosis in 6 children, mild chronic hepatitis with mild fibrosis in 2, minimal chronic hepatitis with moderate fibrosis in 2, minimal liver chronic hepatitis with severe fibrosis in 1, and mild chronic hepatitis with severe fibrosis in the remaining child. Baseline and final features of the children are shown in Table I.

Serological Markers

HBsAg, HBeAg, anti-HBe, anti-HBs, and anti-HDV were tested by commercial radioimmunoassays (Abbott Laboratories, North Chicago, IL). Anti-HIV-1 and anti-HCV were tested by commercial enzyme-linked immunoassays (Abbott and Ortho Diagnostics System Inc., Raritan, NJ, respectively). Liver function tests were analyzed by standard methods (Smac 20, Technicon, NY).

Serum HBV-DNA was detected by dot-blot hybridization, according to the method of Berninger et al. [1982].

HBV-DNA Amplification and Direct Sequencing

Viral DNA was isolated from 200 μl of serum after proteinase K (1 mg/ml) and 1% sodium dodecyl sulfate (SDS) digestion at 37°C for 2 hr, and from liver tissue after homogenization with TE buffer (10 mM Tris pH 7.5, 10 mM ethylenediamine tetraacetic acid [EDTA]) and digestion with proteinase K (5 mg/ml), 1% SDS, 40 mM NaCl, 10 mM Tris-HCl pH 7.5, 2 mM EDTA (37°C, 2 hr). Nucleic acids were extracted with phenol, phenol:chloroform (1:1), and chloroform and, after precipitation with 2 M ammonium acetate and ethanol, pellets were dissolved in 20 μl of sterile distilled water. The total DNA concentration was determined measuring its absorbance at 260 nm.

Polymerase chain reaction (PCR) was carried out in duplicate for each sample in a 50-μl-assay containing 5 μl of serum DNA sample or 1 μg of total liver DNA sample, for 35 cycles (94°C for 1 min, 55°C for 1 min,

TABLE I. Basal and Final Features of the Children

		Basal samples				Final samples			
				HBV-DNA PCR				HBV-DNA PCR	
Pat.	Treatment	HBeAg+/ antiHBe+	ALT (IU/l)	serum	liver	HBeAg+/ antiHBe+	ALT (IU/l)	serum	liver
1	IFN-alpha IFN-beta	+/–	195	+	+	–/+	21	+	+
2	–	+/–	153	+	+	–/+	28	+	+
3	IFN-alpha	+/–	159	+	+	–/+	34	+	+
4	–	+/–	316	+	+	–/+	29	+	+
5	IFN-alpha	+/–	58	+	+	–/+	38	+	+
6	–	+/–	68	+	+	–/+[a]	20	–	+
7	IFN-alpha	–/+	235	+	+	–/+	33	+	+
8	IFN-alpha	+/–	660	+	+	–/+[a]	22	–	+
9	IFN-alpha	+/–	99	+	+	–/+	36	+	+
10	IFN-alpha IFN-beta	+/–	58	+	+	+/–	57	+	+
11	–	+/–	191	+	+	–/+	17	+	+
12	IFN-alpha IFN-beta	+/–	151	+	+	–/+	26	+	+

HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus; PCR, polymerase chain reaction.
[a]Patients 6 and 8 were antiHBs+ in the final samples.

68°C for 1 min). The outer primer sequences were 5′-CTGGGAGGAGTTGGGGGAGGAGATT-3′ at nt. position 1730–1755 and 5′-GCGCATCATTTTGCGGGTCACC-3′ at nt. position 2806–2827; and the inner primer sequences for the nested PCR were 5′-GAGGCTGTAGGCATAAATTGGTCT-3′ at nt. position 1777–1801 and 5′-GCCCCGTAAAGTTTCCCACC-3′ at nt. position 2477–2496 [Ono et al., 1983].

The contamination prevention measures described by Kwok and Higuchi [1989] were followed to avoid false-positive results. Appropriate negative controls were included in each PCR assay and each sample was tested twice by different workers in independent experiments; 100% concordance was obtained in all of the cases.

Amplified DNA was purified with QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and Cy5 direct sequencing of the precore/core region products was performed using ALF™ express DNA automated Sequencer (Pharmacia Biotech AB, Uppsala, Sweden).

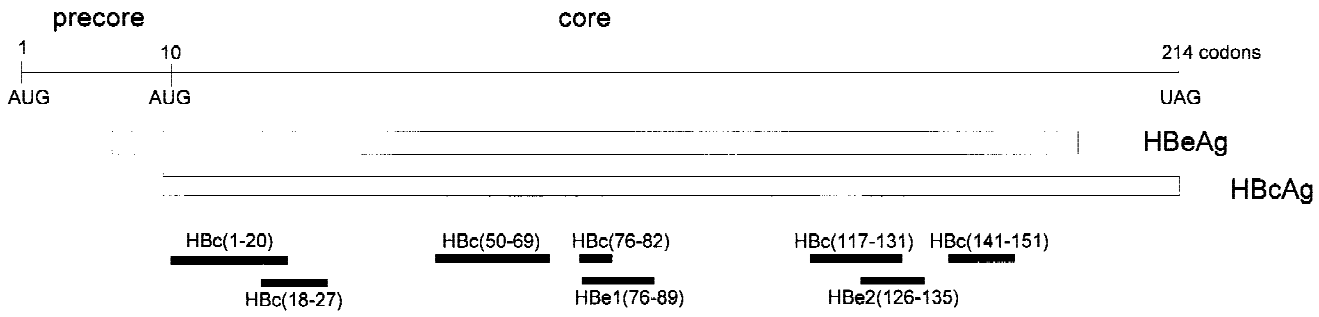
Changes seen in serum and liver samples were correlated with known CD4-restricted Th and CD8-restricted CTL core epitopes, and with described anti-HBc and anti-HBe B cell epitopes, which are represented in Figure 1.

RESULTS

In the baseline serum samples, 11 children had HBV-DNA dot-blot hybridization and one had HBV-DNA detectable only by PCR; the basal liver samples were HBV-DNA positive by PCR in all patients. When the second samples were analyzed, all but one child were responders to the IFN therapy; the HBV-DNA was undetectable by dot-blot hybridization, however. Viral DNA in serum was detected by PCR in all but two children (patients 6 and 8), but all patients had PCR detectable HBV-DNA in their second liver biopsies.

In the precore region of HBV genome, the point mutation (G to A) at nucleotide position 1896 that generates a stop codon was detected together with the wild-type sequence in the viral genomes isolated from the basal serum and biopsy samples of 5 (42%) of the 12 children. The remaining patients had wild-type precore sequences in both serum and liver samples. In the second serum and liver samples, no changes in the distribution of precore sequences were found with respect to that observed in the baseline samples, except in one case (patient 5). In patient 5, a mixture of wild-type and precore 1896 mutant sequences was detected in the baseline samples, but only wild-type precore HBV was found in the second serum and liver samples (Fig. 2).

Only the wild-type sequence of the core gene region was found in the baseline and final serum and liver samples analyzed in three children (patients 3, 6, and 7). In 6 other patients (patients 1, 4, 9, 10, 11, and 12) both the wild type sequence and viral genomes harboring nucleotide changes, which generate amino acid substitutions in the core protein, were found in the baseline serum and/or liver samples (Fig. 2). Most of the amino acid changes found in the baseline samples remained detectable in the second serum and liver samples from these patients, except in patient 10, in whom only wild-type virus was recovered in these second samples (Fig. 2). In two other children (patients 5 and 8) only wild-type HBV was found in the baseline serum and liver samples. Neither of the mutations was detected in the second serum sample; however, in the final biopsy, core sequences with amino acid substitutions at positions 17 and 25 (patient 5) and 42 (patient 8) were detected in addition to the wild-type virus. Finally, the wild-type sequence was found in patient 2 in both serum samples. However, when the HBV-DNA amplification was carried out on the baseline liver sample, a shorter PCR fragment was detected in addition to the expected size product. The sequencing indicated that the shortened form observed in this sample



Epitope (aa residues)	Type of cell epitope	Reference
HB core (76-82)	B	Salfeld 1989, Sällberg 1991a, Schödel 1992
HB e1 (76-89)	B	Salfeld 1989, Sällberg 1991a, Seifer 1993
HB e2 (126-135)	B	Sällberg 1991b, Seifer 1993
HLA class I restricted		
HB core (18-27)	CTL (HLA-A2)	Bertoletti 1991, 1993, Penna 1991
HB core (141-151)	CTL (HLA-A31/AW68)	Missale 1993
HLA class II restricted		
HB core (1-20)	Th *	Ferrari 1991, Jung 1994
HB core (50-69)	Th *	Ferrari 1990, 1991, Jung 1994
HB core (117-131)	Th (DRW52/DR6)	Ferrari 1991, Jung 1994

Fig. 1. Schematic representation of the cytotoxic T-lymphocytes (CTL), T helper (Th) and B cell epitopes sequences within the hepatitis B core and e antigens. *These epitopes are considered promiscuous for various Human Leucocyte Antigen (HLA) class II haplotypes.

represented a deletion within the core gene that affects amino acids 30–177. In the second liver sample, this deletion mutant was not found and only wild-type virus was recovered.

Most of (16/24, 67%) the 24 changes found in the core amino acid sequence were in the hepatitis B core T- or B-cell epitopes described (Figs. 1 and 2). The amino acid 147 deletion in the baseline biopsy sample of patient 2 affected 5 of these 7 epitopes. Regarding the clinical outcome, the presence of core mutations before treatment was not associated with a lower response to the therapy or seroconversion to anti-HBe. In addition, the treatment does not induce any specific or higher number of core mutations.

DISCUSSION

A potent polyclonal and multispecific T-cell response is elicited during acute self-limited HBV infection and is believed to be the cause of the virus clearance [Ferrari et al., 1991; Bertoletti et al., 1993; Missale et al., 1993; Chisari and Ferrari, 1995]. On the other hand, although IFN therapy produces ALT normalization and anti-HBe seroconversion in approximately 50% of the patients with chronic HBV infection, most of them continue to have PCR detectable HBV-DNA in serum [Di Bisceglie, 1995; López-Alcorocho et al., 1997]. The IFN effect in chronic hepatitis B patients is mediated by both direct antiviral activity and enhancing the host immune response [Sen and Ransohoff, 1993; Fang et al., 1994]. It may be hypothesized that viral persistence after ALT normalization might be due to the appearance of mutants in the precore and core regions of the HBV genome, especially in the different immunodominant epitopes identified for the B and HLA class I- and II-restricted T cells [Bertoletti et al., 1994; Carman et al., 1997]. Because the location of HBcAg B-cell epitopes is not accepted universally, we sequenced the complete precore-core sequence of the HBV genome isolated from serum and liver samples taken before and after the IFN treatment from 12 children with chronic hepatitis B and analyzed all proposed AgHBc B- and T-cell epitopes to test this hypothesis.

A previous report [Carman et al., 1997] concerning changes in the precore-core region in spontaneous anti-HBe seroconverters found a temporal association between changes in the core protein and the appearance of the 1896 nucleotide mutant in the precore region. However, this association has not been observed in our population of IFN-induced seroconverters. The reason for this discrepancy is not clear, but it may be related to the different seroconversion mechanisms in the two populations, this being immune mediated in the case of

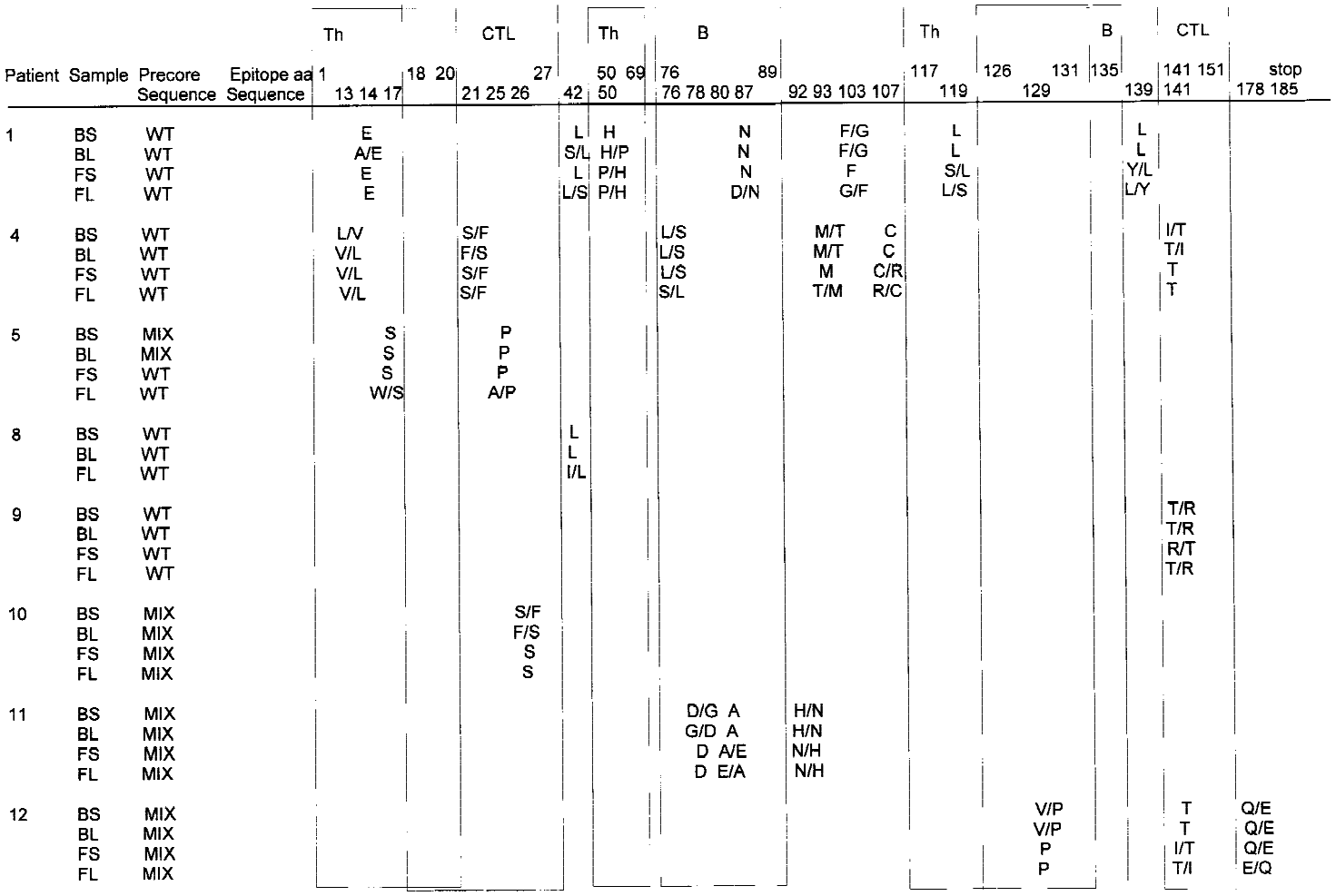


Fig. 2. Distribution of amino acid (aa) substitutions in basal and final samples from the children. Bordered areas indicate the B, T helper (Th), or T cytotoxic (Tc) cell epitopes, with aa boundaries at the top. Amino acids are described by the single letter code. Only the positions in which changes have occurred are shown; when there is a mixture of wild type and mutant sequences in a sample, the species having the greatest proportion is indicated first. BS, basal serum sample; BL, basal liver sample; FS, final serum sample; FL, final liver sample; WT, wild type precore sequence; MIX, mixture of wild type and 1896 precore mutant sequences.

spontaneous seroconversion and immune and direct antiviral effect mediated in the case of IFN seroconverters. Finally, the discrepancy may be a reflection of the prevalence of distinct HBV genotypes in different countries, as it has been suggested that the different HBV genotypes have distinct susceptibility to the 1986 precore mutation [Bagci et al., 1996].

On the other hand, we observed that most of the precore-core sequences found in the basal serum and liver samples when the patients were HBeAg positive and had a high viral replication level were identical to the sequences found in the second samples taken after IFN treatment when the patients were anti-HBe positive and had low viremia levels. This finding shows that all HBV variants found in our patients are equally sensitive to IFN and that the viral persistence after IFN therapy is not due to the appearance of HBV mutants in the B- and T-cell epitopes of the precore-core region of the viral genome. Furthermore, these amino acid changes do not seem to affect the viral replication per se, because the same sequences were found when the patients had high and low viremia levels.

Another interesting finding is that the comparison of the core gene sequences between serum and liver samples shows that almost all sequences detected in the liver were also found in the serum. This result indicates that all HBV variants detected in serum are replication competent or they are complemented in *trans* by the wild-type virus.

Finally, no relationship between the changes found in the precore-core sequences and the clinical outcome of the disease was observed, since all but one child responded to the IFN treatment and their ALT levels have remained normal in all patients to date.

In conclusion, it was found that the viral persistence after response to IFN therapy is not due to the emergence of mutants in the T- and B-cell epitopes of the precore-core region of HBV genome. The results suggest that once IFN reduces the HBV replication levels, the host immune response can control the viral replication, maintaining the low viral replication levels. However, viral persistence due to the appearance of mutants in other HBV genes cannot be discarded.

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